Editorial

Does the *Caenorhabditis elegans* protein CED-4 contain a region of homology to the mammalian death effector domain?

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The elucidation of the apoptosis pathway in the nematode *C. elegans* has been helpful to understand apoptosis signaling pathways in higher eukaryotes. In the worm three genes *ced-3*, *-4* and *-9* are involved in regulating the execution of apoptosis during development (Ellis *et al*, 1991). *Ced-3* codes for a protease homologous to the caspase family. CED-3 activity is negatively regulated by CED-9 which is homologous both structurally and functionally to Bcl-2 and other Bcl-2 family members (Hengartner *et al*, 1992) such as Bcl-x_L. To exert its function CED-9 requires the presence of CED-4, another apoptosis promoting factor (Shaham and Horvitz, 1996). A mammalian CED-4 homolog has not been identified yet. Several reports showed that the CED-3 homolog CPP32/caspase 3 and other CPP32-like caspases act downstream of the CED-9 homolog Bcl-2 (Chinnaiyan *et al*, 1996; Armstrong *et al*, 1996; Shimizu *et al*, 1996; Perry *et al*, 1997; Estoppey *et al*, 1997). In analogy to the sequence of interactions in *C. elegans* the mammalian CED-4 homolog would then be expected to act in between Bcl-2 and a CPP32-like caspase. This scenario got more complex with the identification of another member of the CPP32-like caspases, FLICE/Mach/Mch5/caspase-8 (Muzio *et al*, 1996; Boldin *et al*, 1996; Fernandes-Alnemri *et al*, 1996), that couples directly to the activated CD95 receptor through binding to FADD/MORT1 (Muzio *et al*, 1996; Boldin *et al*, 1996). Could CED-4 act upstream of FLICE, the most receptor-proximal caspase?

Nagata (1996) published a homology between the first FLICE death effector domain (DED) and CED-4 placing the region of weak homology into the CED-4 C-terminus. Recently, another homology alignment between the DED of FLICE with the N-terminus of CED-4 was published (Bauer *et al*, 1997). Regardless of the location of the putative homology box with the DED in CED-4 these data stimulated the idea that the N-terminus of FLICE either functionally played the role of CED-4 in the mammalian system or directly bound mammalian CED-4.

Recently, a number of reports demonstrated that CED-9 can physically interact with CED-4 (Spector *et al*, 1997; Wu

et al, 1997; Chinnaiyan *et al*, 1997; Otilie *et al*, 1997). This interaction was also found using the mammalian CED-9 homolog Bcl-x_L. In addition, Chinnaiyan *et al* (1997) provided a molecular framework involving a physical association of CED-4 with ICE or FLICE. The authors presented evidence that Bcl-x_L could physically interact with FLICE. This interaction was indirect and required the presence of a cellular protein x, assumed to be a mammalian CED-4 homolog. We have recently shown that CD95 apoptosis resistance in peripheral T cell populations is characterized by a lack of FLICE recruitment into the CD95 death-inducing signaling complex (DISC) and upregulation of Bcl-x_L (Peter *et al*, 1997). Taking together all data, Bcl-x_L might directly regulate FLICE activity through binding to the FLICE-N-terminus. This would be in line with the order of events in *C. elegans*. Experiments based on this assumption were in part provoked by the weak homology reported between the FLICE-N-terminus and CED-4. Recent data from our laboratory and a number of inconsistencies now prompt us to put this model in doubt:

1. Association between Bcl-x_L and FLICE was also found with ICE (Chinnaiyan *et al*, 1997). Since ICE does not contain a DED it is unlikely that the DED structure is required for CED-4 binding. In addition, association with CED-4 required either the N-terminal or the C-terminal part of ICE or FLICE. This also does not support the idea of the presence of a specific DED-related binding motif in both caspases.
2. Golstein (1997) has suggested that the CED-9 homolog Bcl-x_L plays a fundamental role by interfering with the CED-3 homolog FLICE while bound to mitochondria. If this were the case this effect should be most pronounced in CD95-mediated apoptosis, in which FLICE plays an essential role.

Recently, we have described the mechanism of FLICE activation in CD95-mediated apoptosis (Medema *et al*, 1997a). Activation of FLICE depends exclusively on recruitment to the CD95 receptor. Therefore, if CED-4 would play a role in the activation of FLICE this would have to occur within the DISC and not at the mitochondria. A mammalian CED-4 homolog would then be expected to be part of the DISC. Furthermore, since Bcl-x_L/CED-9 was shown to directly interact with CED-4, it might also directly bind to the DISC thereby inhibiting the activation of FLICE. To experimentally test this possibility we looked for a cell line that was rendered resistant to CD95-mediated apoptosis by

Figure 1 Alignment of known death effector domain (DED) structures (Thome *et al.* 1997) including two published CED-4/death effector domain homology regions and the murine proteins FADD (GenBank accession number: U43184) and PEA-15 (GenBank accession number: X86694). Black boxes indicate $\geq 50\%$ identity and grey boxes indicate $\geq 50\%$ sequence similarity by conserved amino acids replacements. In addition two new putative DED containing proteins are shown (GenBank Accession numbers: Aa124451 and W23795). The two putative CED4 DED regions (Nagata, 1996; Bauer *et al.* 1997) are boxed

overexpression of Bcl-2 or Bcl-x_L. However, in most reports this does not substantially affect CD95 apoptosis sensitivity (Strasser *et al*, 1995, Itoh *et al*, 1993; Martin *et al*, 1995; Enari *et al*, 1995; Memon *et al*, 1995; Huang *et al*, 1997). Some reports even placed the action of Bcl-2/Bcl-x_L in an apoptosis pathway parallel to the CD95 pathway (Chinnaiyan *et al*, 1996; Strasser *et al*, 1995). One of the few cell lines in which overexpression of Bcl-2 or Bcl-x_L significantly delays CD95-mediated apoptosis are MCF7-Fas cells (Jäättelä *et al*, 1995). Testing the MCF7-Fas cells we found that overexpression of Bcl-x_L in these cells did not affect FLICE recruitment to the DISC or its activation (Medema *et al*, 1997b). Since it was published that Bcl-x_L could be coimmunoprecipitated with FLICE when transiently transfected into 293T cells (Chinnaiyan *et al*, 1997) we tested whether Bcl-x_L/FLICE association could be found in MCF7-Fas cells stably expressing Bcl-x_L. Using either antibodies against Bcl-x_L or a set of monoclonal antibodies against different domains of FLICE we did not detect any association of Bcl-x_L with FLICE or its active subunits in these cells (unpublished observations). In addition we did also not find any binding or functional inhibition of the active FLICE subunits p18/p10 by Bcl-x_L. Taken together this makes it difficult to place the action of CED-4 upstream of FLICE.

3. Both published alignments between CED-4 and the mammalian DED, one placing the homology box at the CED-4 C-terminus (Nagata, 1996), the other at its N-terminus (Bauer *et al*, 1997) were based on the rather limited number of sequences of FLICE, FADD and PEA-15, a phosphoprotein of as yet unknown function found in astrocytes (Muzio *et al*, 1996). Thome *et al* recently reported the identification of a new class of viral FLICE inhibitory proteins (v-FLIPs) that are characterized by the presence of two DED (Thome *et al*, 1997). In this paper we demonstrated that these proteins both functionally and physically interact with FLICE and the DISC confirming that the search

algorithm was used identified functional DED containing proteins. Using this algorithm two new DED containing proteins were identified (Figure 1). One of these proteins (Accession number: W23795) shares 41% identity on the amino acid level within the DED1 of the MCV 160L and might therefore represent a human homolog of v-FLIP. Using this extended list of known DED motifs the CED-4/DED homology can be put to the test (Figure 1). Comparison of 22 different DED reveals the main conserved amino acid positions. Compared to this alignment the region in the CED-4 N-terminus published to be homologous to the DED region by Bauer *et al* (1997) does not contain these key residues. In addition the alignment by Nagata (1996) involving the C-terminus of CED-4 does not cover the complete motif.

This analysis demonstrates the pitfall to only rely on a weak homology without functional data to substantiate it. Even the availability of such functional data does not guarantee the significance of an alignment. This is exemplified by the apoptosis-inducing *Drosophila* protein reaper (White *et al*, 1994). Reaper was suggested to contain a death domain (DD) by sequence alignment with the CD95- and TNF-RI-DD (Golstein, 1995). However, mutagenesis of reaper and functional experiments in both mammalian and insect cells demonstrated that the essential amino acid positions found at the N-terminus of the TNF-RI DD could be replaced in reaper without affecting its cytotoxic activity (Chen *et al*, 1996; Vucic *et al*, 1997). In addition, a reaper DD structure is inconsistent with the NMR structure of the CD95 DD as reported and pointed out by Huang *et al* (1996). The situation with the CED-4/DED homology seems to be similar. Taking together all arguments, it is unlikely that CED-4 contains a motif structurally homologous to the mammalian death effector domain. Thus, the CED-9 homolog Bcl-x_L seems to act downstream of FLICE and CED-4 might have a different function than binding to DED containing proteins.

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